STUDIES ON SIALIC-ACID-CONTAINING GLYCOPROTEINS

BY

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Studies on Sialic-acid-containing Glycoproteins

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This paper is a summary of the following communications:

I. ODIN, L.: Carbohydrate residue of a urine mucoprotein inhibiting influenza virus haemagglutination. *Nature 170*, 663, 1952.
II. ODIN, L.: Sialic acid in pseudomyxomatous gels. *Acta Chem. Scand. 9*, 714, 1955.
III. ODIN, L.: Sialic acid in human serum protein and in meconium. *Acta Chem. Scand. 9*, 862, 1955.
IV. ODIN, L.: Sialic acid in human cervical mucus, in hog seminal gel, and in ovomucin. Acta Chem. Scand. *9*, 1235, 1955.
V. BERGGÅRD, I. and ODIN, L.: Studies on the transformation and degradation of sialic acids in aqueous solution. *Arkiv f. Kemi 12*, 581, 1958.
VI. ODIN, L.: Studies on the chemistry of ovarian cyst contents. *Acta Soc. Med. Upsal. 64*, 25, 1959.

References to these papers will be made with Roman figures I–VI.

The carbohydrate groups of animal glycoproteins are of many different types. Apart from uronic-acid- and ester-sulphate-containing materials, which are not discussed, the carbohydrate moieties are composed of one or two kinds of hexosamine, in combination with usually two different neutral sugars or with sialic acids or with both. The hexosamines are N-acetyl-glucosamine and N-acetyl-galactosamine; the neutral sugars are galactose, mannose, and fucose; and N-acetyl-, N-glycolyl-, N,O-diacetyl-, and N-acetyl-O-diacetyl-neuraminic acid are the sialic acids hitherto identified (4, 5). Whether or not the glycoproteins display acid properties seems largely to depend on the sialic acid content. Essentially neutral are most preparations of purified blood-group substances and the main glycoproteins of many mucous epithelial secretions, the neutral sugar constituents of which are galactose and

fucose occurring in roughly equal quantities or with galactose predominating. (See e.g. 20, 42.) Varying but usually small amounts of sialic acid are also present. These glycoproteins have been termed fucomucins (3). Another type of neutral glycoprotein is represented by the ovomucoid of egg-white, which seems to contain glucosamine, mannose, and galactose in the approximate ratio 7:3:1 (34). Markedly acid properties on the other hand are displayed by the secretions of the salivary glands. Submaxillary mucins thus contain equimolecular amounts of sialic acid and hexosamine, but inconsiderable amounts of neutral sugar (2, 6, 30). This type of glycoprotein may be called sialomucin. In addition there are glycoproteins that contain appreciable amounts of both neutral sugar and sialic acid. Some acid plasma glycoproteins belong to this group (see 45). Their neutral sugars are galactose and mannose in approximately equal quantities, together with smaller amounts of fucose. Some epithelial mucous products, e.g. human urinary mucin, cervix-uteri mucus, meconium, the contents of certain pseudomucinous cystadenomata, boar-seminal gel, and the ovomucin of hen's egg-white, also seem to contain a 'mixed' type of glycoprotein. Their carbohydrate constituents will be discussed below.

During recent years considerable attention has been paid to glycoproteins rich in sialic acid, because of their property of inhibiting influenza-virus haemagglutination. This property has recently been shown largely to depend on the presence of sialic acid, which probably acts as a receptor group for the virus (see 14).

The present work is mainly devoted to the study of the carbohydrate group of materials containing the 'mixed' type of glycoprotein.

Analytical methods (VI)

Most of the analytical principles employed are in common use. A few comments are warranted, however.

The content of *total neutral sugar* was assayed by the Tillmanns-Philippi orcinol-sulphuric acid reaction in Vasseur's modification (40). This is particularly well suited for this purpose, as the absorbancy at the wavelength used, 490 mμ, is approximately the same for all the neutral sugars present. The high sample-blank absorbancies obtained with materials containing little carbohydrate make sample-blank corrections advisable. Such correction may give slightly too low carbohydrate figures, however, as the orcinol present probably inhibits part of the chromogen formation caused by the acid.

In the Dische cystein-sulphuric acid reaction for *methylpentose* (9) there was a marked difference between materials rich and poor in fucose, in the time taken after the addition of cystein to reach constant absorbancy. The relationship between the contents of fucose and preformed cystein may have some bearing on this phenomenon. As maximum

absorbancy was not reached with the fucose-rich specimens until after 4 hours (44) this time interval was allowed to elapse before making the readings.

In agreement with Laurent's findings (25) it was observed that the results of the orcinol and cystein reactions did not differ appreciably between determinations made on previously hydrolysed and on unhydrolysed specimens respectively.

For the estimation of the *relative contents of the individual neutral sugars* a simple procedure with satisfying accuracy was worked out. This included acid hydrolysis, paper chromatographic fractionation, and quantitative assay of the eluted monosaccharides. Similar methods have been used by others (see 19). In the case of the various kinds of cyst content and egg-glycoproteins the method by which maximum quantities of all neutral sugars could be split off was found to be two hour's heating at 100°C in 2 *N* sulphuric acid. In the cyst material fucose, when present, seemed to be liberated at a somewhat higher rate, and mannose at a lower rate, than galactose. This observation corresponds to earlier findings with other materials such as serum proteins and blood-group substances (see 32). The sugars were recovered in a yield of 70–80 % of the amounts found in the unfractionated material by the orcinol method, both from solutions of pure monosaccharides and from hydrolysates of cyst contents. Some losses occurred in the fractionation and elution steps, but the low yields may partly be explained by changes occurring in the sugar molecules on heating with acid. These products may still be capable of reacting with the orcinol and cystein reagents, however, since the results obtained by these reactions (performed on unfractionated material) are approximately the same with and without previous hydrolysis, as mentioned above.

Sialic acid was determined by the Bial-orcinol-Fe^{+++} reaction. The procedure of Werner and Odin (43) was mainly followed, except for the heating time, which was changed to 15 minutes in accordance with Svennerholm's technique (36). This was done because maximum absorbancy was not achieved with sialic-acid-containing proteins (in contrast to the free acids) until after this period. Correction for non-specific absorbancy was found to give almost identical results when performed by dichromatic readings (36) and simple subtraction (43). For materials, such as isolated fucomucins, with very high neutral-sugar : sialic-acid ratio the most reliable results were probably those obtained by running standards containing the sugars in approximately the same proportions as in the material to be tested.

Isolation and identification of sialic acid (II–IV, VI)

For the liberation of sialic acid, Blix's method (2, 5) of heating the suspended or dissolved iso-electric material in water at 100°C was

usually employed, but in some cases the hydrolysis had to be done with dilute sulphuric acid (about 0.01–0.02 *N*). The former technique was preferred when possible in spite of the lower yields, in order to avoid the splitting off of any O-acetyl groups present and to avoid further degradation. The crystallization of the sialic acid split off was performed either directly (2, 5) or after adsorption onto an anion exchange resin (35, 47). The latter technique was advantageous when the sialic acid was much contaminated with other split products. The importance of removing inorganic material, and particularly cations, before crystallization is emphasized.

The isolated product was invariably identified by X-ray diffraction, and in some cases also by paper chromatography and chemical analysis.

All sialic acid isolated from material of human, porcine, and avian origin, was identified as N-acetyl-neuraminic acid. This was noteworthy, since at the time the experiments were carried out, sialic acids that had been isolated from different animal species all differed in their substituent groups. Since then has been demonstrated that more than one variety of sialic acid may be present in the same material (Klenk *et al.* (e.g. 22); Svennerholm *et al.* (e.g. 26, 37)), and that X-ray findings may be misleading, since the sialic acids are capable of forming solid solutions of each other (1). Thus, besides N-acetyl-neuraminic acid, other sialic-acid varieties may be present in some of the material under review. So far, human material has been found to contain only N-acetyl-neuraminic acid, however.

Transformation and degradation of sialic acids in aqueous solution (V)

The effect of pH and temperature on solutions of various sialic acids was studied by examination of the ultraviolet absorption and by paper chromatography. At room temperature the sialic acid showed a simple end-absorption. The methyl esters when dissolved at a neutral or moderately alkaline reaction displayed transient absorption with the maximum at about 265 mμ. This absorption was interpreted as being due to enolization of the open-chain carbonyl form. The ester group seemed to favour the enol formation, as has been shown to be the case with acetoacetic acid (18). The disappearance of the absorption would then be due to hydrolysis of the ester. On heating, the sialic acids showed a maximum stability at pH 4–5, to judge from the absence of absorption bands and chromatographically demonstrable degradation products. Above and below this pH degradation occurred, N-glycolyl-neuraminic acid appearing to be the most labile of the sialic acids tested, and the N-acetyl derivative the least labile. The degradation occurring at pH < 4 revealed by the appearance of some spots on chromatography and an ultraviolet absorption at 270 mμ indicated that, even at the moderately acid pH usually employed for the splitting off of bound sialic acid,

some decomposition occurs. Heating at pH > 5 resulted in the formation of serveral degradation products as demonstrated chromatographically, among which were the earlier recognized N-acetyl-hexosamine and 2-carboxypyrrole (see 15). On mild alkali treatment in addition to N-acetyl-hexosamine three further chromogens appeared, which were also formed on similar treatment of N-acetyl-glucosamine, and which are probably identical to the Ehrlich-positive chromogens analysed by Kuhn and Brossmer (23, 24). These authors assumed one of these chromogens to be an anhydro-hexosamine, and another was identified as 5-dihydroxyethyl-3-acetaminofuran. On treatment with stronger alkali these chromogens disappeared, and at least three further acid products reacting with the Ehrlich and Bial reagents and showing strong absorption at about 260 mμ, were formed from the sialic acids (but not from N-acetyl-hexosamine). One of the chromogens corresponded to 2-carboxypyrrole on chromatography; the other two are so far unidentified.

The formation of 2-carboxypyrrole (for which Gottschalk has provided strong evidence (13), apparently without having isolated the substance in pure state, however) was definitely proved by its isolation in crystalline form after adsorption onto an anion-exchange resin and elution by acid. It was identified by comparison of its X-ray diffraction pattern with that of a synthetic specimen.

In one experiment the amount of 2-carboxypyrrole formed from N-glycolyl-neuraminic acid after treatment with 0.1 *N* NaOH at 100°C for 30 minutes was found to be only 3 % of the theoretical. Less than 20 % of the absorption at 260 mμ was found to be due to this substance. The ultraviolet absorption of alkali-treated sialic-acid solutions cannot therefore be used for the determination and identification of 2-carboxypyrrole.

Carbohydrate composition of sialic-acid-containing mucus and orosomucoid (I–IV)

Except for (I) the purpose of these studies was primarily to isolate and identify sialic acid in order definitely to prove the presence of this substance in the materials investigated. Its occurrence had in some cases earlier been indicated by colour reactions.

Urinary mucin (I). The material was prepared as described by Tamm and Horsfall (38, 39), and also by centrifuging at high speed. These preparations all showed a similar composition. The analysis of the mucin obtained by the method reported to give an essentially homogeneous product (39) is given in the table. Similar data (except for the sialic acid) were simultaneously published by Gottschalk (12). As the product obtained by centrifuging does not differ appreciably from the material

Table 1. *Composition of mucus from different sources.*
(Per cent of dry weight when not otherwise stated.)

Material	N	Hexosamine			Neutral sugar					
			Per cent of total				Per cent of total			
		Total	Glucos-amine	Galactos-amine	Hex-ose	Fuc-ose	Galac-tose	Man-nose	Fuc-ose	Siali acid
Urinary mucin	10.9	9.4	87	13[a]	8.4	1.1	59	29	12[a]	9.1
Cervical mucus	9.7	10.5	63	37	7.5	5.0	60	tr	40	7.4
Meconium	10.1	11.8			7.9	4.9				5.0
Hog seminal gel	10.7	10.5	20	80	4.0	0.7	58	28	14	14.7
Ovomucin	13.3	7.1	74	26	6.6	0	67	33	0	6.0
'Pseudomyxomatous' ovarian cyst content no. 34 (VI)	8.9	15.6	52	48	10.8	3.5	60	10	30	17.5
'Pseudomucinous' ovarian cyst content no. 30 c (VI)	7.9	18.7			16.2	18.3	49	tr	51	1.0

[a] Odin (30).

isolated by the Tamm-Horsfall procedure, this glycoprotein probably forms the main part of the so-called nubecula, i.e. the mucous 'cloud' appearing in urine on standing.

Pseudomyxomatous gels (II). The gelatinous contents of certain pseudomucinous (pseudomyxomatous) ovarian cystadenomata were found to contain considerable amounts of sialic acid, in contrast to the 'ordinary' pseudomucinous material, which contained small quantities only of this substance. The sialic acid was isolated and identified as N-acetylneuraminic acid. (The composition of the gels is discussed in a later section.)

Cervical mucus (IV). The cervical mucous plugs of pregnant women were dried and analysed (table 1). Similar data have been published by Werner (42). N-acetyl-neuraminic acid was isolated.

Meconium (III). The material was washed with dilute hydrochloric acid. The insoluble matter was analysed (table 1), and N-acetyl-neuraminic acid isolated from it. The carbohydrate constituents of meconium have been identified by Werner (42), who gave no quantitative data, however.

Hog seminal gel (IV). The gelatinous lumps of hog semen were washed, dried, and analysed (table 1). N-acetyl-neuraminic acid was isolated. The bound carbohydrate of this material does not previously seem to have been subjected to chemical analysis.

Ovomucin (IV). The preparation was made by dilution of egg-white with water (16). The composition of this material is given in table 1. Sialic acid was isolated and identified as N-acetyl-neuraminic acid. Galactose and mannose have earlier been identified as the neutral-sugar constituents of ovomucin by Sørensen (33), who gave the ratio 1:1 for these sugars. Hexosamine has been determined by Young (46) and Meyer (27). Glucosamine and galactosamine have been isolated by Odin (29). The presence of sialic acid has been indicated by Werner and Odin (43) by means of colour reactions.

Serum protein (III). A sialic acid was isolated from orosomucoid (see 45) prepared by the method of Weimer *et al.* (41), and from ethanol-precipitated total serum protein, and identified as N-acetyl-neuraminic acid. Sialic acid has been shown to occur in this material, and suggested to be responsible for its acid properties. (Odin and Werner (31).)

Comment. A characteristic feature of all these materials which in other respects differ widely is the comparatively high sialic-acid content. In addition to this substance and hexosamines, varying amounts of neutral sugars are also present. In the epithelial mucous secretions (except for ovomucin), these are galactose, fucose, and in some cases mannose. The materials differ from fucomucin-containing mucus mainly in the high sialic-acid content, and, for some of them, the low fucose content, and in the presence of mannose, the quantity of which in some of the materials would refuse the possibility that it is derived from contaminating serum protein. The carbohydrate group of ovomucin has a quite different composition, and that of orosomucoid represents yet another type, which is also found in other serum proteins and in tissue proteins. All the sugars present in the type of glycoproteins under investigation are probably attached to the same molecule since some of these glycoproteins may be considered homogeneous. The fact that sialic acid is so easily split off, while at the same conditions only inconsiderable amounts of other carbohydrates are liberated, indicates a terminal position for at least part of the sialic acid residues.

Connexion between sialic-acid content and influenza-virus haemagglutination-inhibiting acitivity (I)[1]

It had been shown, notably by Burnet and his group that many 'mucins' inhibit influenza-virus haemagglutination (see 7), and that the 'receptor group' is probably of carbohydrate nature. Gottschalk had shown that a dialysable product was split off by the action of virus on ovomucin (17) and urinary mucin (11). At that time he believed this product to be an *iso*-glucosamine–amino acid complex.

[1] This investigation was made in collaboration with Dr. Carola Neumüller, State Bacteriological Institute, Stockholm, who kindly performed the determinations of inhibitor activity.

In (I) it was shown, that like the urinary mucin, a great many haemagglutination inhibitors contain sialic acid. No quantitative relation was found, however, between the content of sialic acid and the inhibiting activity, which varies considerably between different inhibitors. This was assumed to be due either to structural differences in the carbohydrate group (the sialic acid) or in the rest of the inhibitor molecule. The dialysable products formed by the action of influenza virus on the urinary mucin was found to give strongly positive reactions with Bial's reagent and, even without previous alkali treatment, with Ehrlich's reagent. This was thought to indicate sialic acid (the structure of which at that time was unknown) to be involved in the interaction between virus and inhibitors (or erythrocytes). This suggestion was subsequently confirmed by the actual isolation of the split product and its identification as N-acetyl-neuraminic acid by Klenk, Faillard, and Lempfrid (21). Sialic acids have since been isolated by the action of influenza virus and bacterial enzymes from several inhibitors and from erythrocytes. (For literature see e.g. Faillard (10).) For the recent work by Gottschalk and others on the structure of the split product, viz. the sialic acids, ... see (8).)

The chemistry of ovarian cyst contents (VI)

Because of the findings in (II) referred to above, a more comprehensive study was carried out on the composition and particularly the glycoprotein components of the contents of a variety of ovarian cysts. Special attention was paid to the gelatinous material rich in sialic acid, produced by certain pseudomucinous cystadenomata. These gels are, because of their physical and chemical characteristics (see below), distinguished from the 'ordinary' pseudomucinous material, and are arbitrarily called *pseudomyxomatous*.

'Ordinary' pseudomucinous cyst contents. These were all fluids, although in many cases of extremely high viscosity. (The fluid state may not, however, be an invariable property of this tumour secretion.) They were with few exceptions soluble in water. The characteristic components were essentially neutral glycoproteins containing about 50 % of carbohydrate, and which were of the fucomucin type. The sugar constituents were, accordingly, glucosamine, galactosamine, galactose, and fucose. The sialic-acid content was low, usually not exceeding 2 %. In three cases only, it was higher (up to 3.7 %), and in two of these fluids the proportions of the other sugar constituents were similar to those found in the cyst contents treated in the next section. These fluids thus probably represent an intermediate form of pseudomucinous secretion. The ratio of the sugar constituents is illustrated by the data given in the table for one specimen particularly rich in carbohydrate. In some fluids the presence of at least two fucomucin fractions was indicated. In

these cases the fraction moving fastest on electrophoresis showed a higher sialic-acid content than the slower one. In addition to the glycoproteins, which may occur in low concentration, varying amounts of serum protein were also present in the fluids.

The presence of fucomucins in the pseudomucinous cyst contents is by no means surprising as these fluids are known sometimes to contain large quantities of blood-group substances (which belong to the fucomucin type of glycoprotein) and have been used as sources for the isolation of soluble blood-group antigen (see 28). Furthermore, as mentioned earlier, the main glycoprotein component of many other epithelial mucous secretions is of fucomucin nature. There is in fact no fundamental difference from a chemical point of view, between the contents of these pseudomucinous cysts and most mucous secretions of other origin.

Pseudomyxomatous material. This material included (1) the contents of ovarian cystadenomata classified by the pathologist as pseudomucinous, and (2), associated with the ovarian lesion, (a) the peritoneal gels from patients with pseudomyxoma peritonei and (b) the appendiceal contents from patients with mucocele of the appendix. At least six of the nine ovarian tumours were associated with a pseudomyxoma peritonei. The material formed rigid gels, which, apart from admixed serum protein, were practically insoluble in aqueous solvents within a wide pH-range. The sugar constituents of the gelatinous material were glucosamine, galactosamine, galactose, fucose, and sialic acid, and in addition small amounts of mannose and probably xylose. No difference was found between gels of ovarian, appendiceal and peritoneal origin. An obvious difference from the fucomucin-containing cyst material, discussed above, is the high sialic acid and low fucose contents. The carbohydrate composition of an extensively washed specimen is given in the table.

The gels were brought into solution by treatment with alkali, or with strong urea, or by tryptic digestion. The urea-solubility is suggested to indicate that hydrogen bonds are important for the gel state. When studied electrophoretically the alkaline solutions and the digested material were found to contain at least one fast-migrating glycoprotein, the composition of which was similar to that of the thoroughly washed native gels. Because of their high sialic-acid content these glycoproteins may be called sialomucins. In the alkali-treated material serum proteins, particularly albumin, were also present. Significant amounts of the fucomucin type of glycoprotein did not occur. The similarity between the gelatinous material and some mucous secretions from the urogenital tract is stressed.

Conclusions. The pseudomucinous cystadenoma is histologically characterized by a typical epithelium, and it is mainly upon this basis that the diagnosis is founded. It is, however, obvious that some pseudomucinous cystadenomata run a clinical course very different from that

of most of these tumours. The cysts of this group have a tendency to spontaneous rupture, and are in some way associated with pseudomyxoma peritonei and mucocele of the appendix. The epithelium of the 'atypical' cystadenomata has not been reported histologically to differ from that of the main group.

In the investigation now reported it is shown that two physically and chemically different types of pseudomucinous cyst secretion occur. One is (usually) fluid and water-soluble, and contains the fucomucin type of glycoprotein; the other is gelatinous and insoluble in water and contains sialomucin. Intermediate forms may occur. These differences seem to correspond to the differences in clinical course outlined above. Thus the second, gelatinous, type of secretion occurs in that type of cystadenoma that is commonly associated with pseudomyxoma peritonei. Furthermore, these cyst contents are chemically and physically identical to the peritoneal gels found in the pseudomyxoma, and to the appendiceal contents from coexistent mucocele of the appendix.

In view of these differences, it is suggested that two varieties of pseudomucinous cystadenoma be distinguished (possibly with intermediate forms). On the basis of the chemical findings it is proposed to substitute the term *pseudomucinous* by *mucous* and to call the different varieties of this tumour *fucomucous*, *sialomucous*, and, perhaps, *intermediate-type mucous* cystadenoma.

No conclusions concerning the histogenesis is considered warranted from the chemical findings reported. No obvious connexion between histological malignancy of the tumour and the chemical nature of the secretion is noted.

Serous cyst contents. This material included the contents of many different types of cysts and cystic tumours: simple and papillary serous cystadenoma; papillary cystadenocarcinoma; follicular, lutein, endometrial, and dermoid cysts; cystic fibroma and myoma; and tubo-ovarian and parovarian cysts.

The fluid contents of most of these cysts have for long been described as serous, their protein components being considered as identical or related to serum proteins.

In the present investigation serum proteins were indeed found to be the main high-molecular constituents of the cyst fluids, thus confirming the accepted view. In a few cysts, mostly of non-ovarian origin, some secretion of mucus occurred.

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Literature

1. Abrahamsson, S., and Svennerholm, L., personal communication.
2. Blix, G., *Z. physiol. Chem. 240*, 43 (1936).
3. Blix, G., *Angew. Chemie 62*, 168 (1950).
4 Blix, G., Proc. IVth Internat. Congr. of Biochem., Vienna, Vol. I. (M. L. Wolfrom, ed.) Pergamon Press, London, 1959.
5. Blix, G., Lindberg, E., Odin, L., and Werner, I., *Acta Soc. Med. Upsal. 61*, 1 (1956).
6. Blix, G., Svennerholm, L., and Werner, I., *Acta Chem. Scand. 6*, 358 (1952).
7. Burnet, F. M., *Physiol. Rev. 31*, 131 (1951).
8. Cornforth, J. W., Firth, M. E., and Gottschalk, A., *Biochem. J. 68*, 57 (1958).
9. Dische, Z., and Shettles, L. B., *J. Biol. Chem. 175*, 595 (1948).
10. Faillard, H., *Z. physiol. Chem. 307*, 62 (1957).
11. Gottschalk, A., *Nature 167*, 845 (1951).
12. Gottschalk, A., *Nature 170*, 662 (1952).
13. Gottschalk, A., *Biochem. J. 61*, 298 (1955).
14. Gottschalk, A., *Physiol. Rev. 37*, 66 (1957).
15. Gottschalk, A., *Nature 181*, 377 (1958).
16. Gottschalk, A., and Lind, P. E., *Brit. J. Exp. Path. 30*, 85 (1949).
17. Gottschalk, A., and Lind, P. E., *Nature 164*, 232 (1949).
18. Grossman, P., *Z. physikal. Chem. 109*, 305 (1924).
19. Hough, L., Analysis of Mixtures of Sugars by Paper and Cellulose Column Chromatography, *in* Methods of Biochemical Analysis, Vol. I. (D. Glick, ed.) Interscience Publishers, New York, 1954, p. 205.
20. Kabat, E. A., Blood Group Substances. Academic Press, New York, 1956.
21. Klenk, E., Faillard, H., and Lempfrid, H., *Z. physiol. Chem. 301*, 235 (1955).
22. Klenk, E., and Uhlenbruck, G., *Z. physiol. Chem. 307*, 266 (1957).
23. Kuhn, R., and Krüger, G., *Chem. Ber. 89*, 1473 (1956).
24. Kuhn, R., and Krüger, G., *Chem. Ber. 90*, 264 (1957).
25. Laurent, B., *Scand. J. Clin. Lab. Invest. 10* (1958), suppl. 32.
26. Mårtensson, E., Raal, A., and Svennerholm, L., *Acta Chem. Scand. 11*, 1604 (1957).
27. Meyer, K., Cold Spring Harb. Symp. Quant. Biol. *6*, 91 (1938).
28. Morgan, W. T. J., *Experientia 3*, 257 (1947).
29. Odin, L., *Acta Chem. Scand. 5*, 1420 (1951).
30. Odin, L., Mucopolysaccharides of Epithelial Mucus, *in* Ciba Symp. Chem. & Biol. of Mucopolysaccharides. J. & A. Churchill, Ltd., London, 1958, p. 234.
31. Odin, L., and Werner, I., *Acta Soc. Med. Upsal. 57*, 227 (1952).
32. Sonnet, J., Les glycoprotéines sériques a l'état normal et patologique. Éditions Arscia, Brussels, 1956.
33. Sørensen, M., *Biochem. Z. 269*, 271 (1934).
34. Stacey, M., and Woolley, J. M., *J. Chem. Soc.* 550 (1942).
35. Svennerholm, L., *Acta Soc. Med. Upsal. 61*, 75 (1956).

36. Svennerholm, L., *Arkiv f. Kemi 10*, 577 (1957).
37. Svennerholm, E., and Svennerholm, L., *Nature 181*, 1154 (1958).
38. Tamm, I., and Horsfall, F. L., *Proc. Soc. Exp. Biol. Med. 74*, 108 (1950).
39. Tamm, I., and Horsfall, F. L., *J. Exp. Med. 95*, 71 (1952).
40. Vasseur, E., *Acta Chem. Scand. 2*, 693 (1948).
41. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem. 185*, 561 (1950).
42. Werner, I., *Acta Soc. Med. Upsal. 58*, 1 (1953).
43. Werner, I., and Odin, L., *Acta Soc. Med. Upsal. 57*, 230 (1952).
44. Wessler, E., and Werner, I., *Acta Chem. Scand. 11*, 1240 (1957).
45. Winzler, R. J., Glycoproteins of Plasma, *in* Ciba Symp. Chem. & Biol. of Mucopolysaccharides. J. & A. Churchill, Ltd., London, 1958, p. 245.
46. Young, E. G., *J. Biol. Chem. 120*, 1 (1937).
47. Zilliken, F., Braun, G. A., and György, P., *Arch. Biochem. Biophys. 54*, 564 (1955).